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13. ABSTRACT ***(Maximum 200 Words)***

Intimate links between the morphology changes of some of sub-nuclear structures and diseases such as cancer have been shown. Thus far, a number of pre-mRNA splicing factors, RNA polymerase II and kinases as well as several cancer related proteins have been reported to be localized to the IGCs. The present study sets a precedent for the global identification of potential differences in the protein composition of IGCs from breast cancer cell nuclei versus normal cells. We have successfully identified the entire protein composition of the isolated IGC fraction from the mouse liver nuclei by proteome analysis using liquid chromatography and tandem mass spectrometry (LC-MS/MS) and combining peptide sequence analysis and database searching. Interestingly, several proteins were identified with well characterized roles in pre-mRNA splicing and yet others known to have structural roles in cells. In addition, we have established the approach to purify IGCs from suspension tissue culture cells, HeLa. The quality of the IGC fraction from the suspension culture has been confirmed using immunoblot analysis. Further, it was observed that the proteins that were enriched in mouse liver IGCs were also found to be enriched in HeLa IGC fractions (splicing factors and RNA processing factors). Work is underway to purify and characterize the IGCs from breast cancer cell lines, HTB 123. Based on these studies it is likely that additional IGC proteins will be identified that have an altered organization and/or abundance in breast cancer cells that may lead to altered patterns of gene expression in these cells.

14. SUBJECT TERMS
Interchromatin granule cluster, gene expression, nuclear speckle, nuclear domain, Pre-mRNA splicing, nuclear compartmentalization

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Table of Contents

Although the start date of this grant is indicated as January 1, 2001, I was appointed as Principal Investigator of the grant on July 1, 2001 as per Modification No. P00001 to Grant No. DAMD17-00-1-0209 (letter from Blossom J, Widder, dated June 25, 2001). Therefore, this annual progress report actually represents work accomplished from July 1, 2001 to December 31, 2001.

INTRODUCTION

Interphase nuclei are thought to be compartmentalized into a highly organized structure to allow efficient nuclear metabolism, such as transcription, RNA splicing, DNA replication, DNA repair/recombination, assembly of ribosomal sub-units, nucleocytoplasmic protein/RNA trafficking, and so on (Lamond and Earnshaw, 1998; Spector, 1993). A large number of subnuclear compartments have been identified in normal and cancer cells, such as IGCs (interchromatin granule clusters, PF (perichromatin fibrils), nucleoli, cajal bodies, GEMs (gemini of cajal bodies), PML (promyelocytic leukemia) bodies, etc. (Spector, 1993; 2001). Active investigations are underway to elucidate roles of these subnuclear compartments in various functions. Intimate links between the morphological changes of some of these structures and diseases such as cancer have been shown (Dyck et al, 1994; Ochs et al, 1994). One of the most interesting nuclear sub-structures, the IGCs is suggested to have a role in establishing and efficiently coupling transcription and pre-mRNA splicing in nuclei (Spector, 1993; Misteli, 2000). The IGCs are enriched in splicing factors, but it has been clearly shown that they are not the site of splicing or transcription *per se*. IGCs are thought to be the sites of complex formation, protein modification and/or storage for factors involved in transcription and splicing. Thus far, a number of pre-mRNA splicing factors, RNA polymerase II and kinases as well as several cancer related proteins have been reported to be localized to the IGCs. One isotype of WT1, Wilms tumor suppressor gene product, that localizes to IGCs in normal cells is not detectable by immunofluorescence from IGCs of breast cancer cells in contrast to normal mammary epithelium (Little et al., 1999; Silberstein et al., 1997). However, the role of this protein in regard to breast cancer remains to be determined. The present study sets a precedent for the global identification of potential differences in the protein composition of IGCs from breast cancer cell nuclei versus normal cells. Based on these studies it is likely that additional IGC proteins will be identified that have an altered organization and/or

abundance in breast cancer cells that may lead to altered patterns of gene expression in these cells.

BODY

As an initial approach to elucidating the protein composition of IGCs from breast cancer cells we have developed a consistent purification procedure using mouse liver nuclei and HeLa cell nuclei as one can easily obtain significant amounts of sample for analysis.

MOUSE LIVER IGC PURIFICATION AND CHARACTERIZATION: We have used two complementary approaches toward identification of the protein composition of IGCs. In the first approach, we resolved IGC protein constituents on 2-D gels as performed previously (Mintz et al., 1999), and excised spots enriched in the purified IGC fraction for peptide microsequencing. In the second approach, we applied the whole IGC fraction to proteome analysis using liquid chromatography and tandem mass spectrometry (LC-MS/MS). This method allowed us to identify the entire protein composition of the isolated IGC fraction by combining peptide sequence analysis and database searching. During the past year we have purified approximately 1 mg of IGCs from mouse liver nuclei for this analysis. As a result, 2214 peptide sequences were obtained from the saturated analysis. From these sequences we identified 128 proteins that were previously known to localize in IGCs or whose functions are associated with IGCs. As expected, we have detected many proteins involved in pre-mRNA processing such as 5'capping proteins, splicing factors, and 3' processing proteins. In addition, we found several sub-units of RNA polymerase II and transcription factors in the IGC fraction. Each of the identified proteins was characterized with regard to its motifs and its localization to nuclear speckles. Our analysis will pick up proteins that are both enriched in IGCs, and therefore localize in a speckled pattern by immuofluorescence microscopy (i.e. snRNPs, SF2/ASF), as well as other proteins that may be equally distributed throughout the nucleoplasm including the IGCs and diffuse nuclear pools (i.e. hnRNP A, C). In addition, we also found proteins that are not well characterized with regard to their sub-nuclear localization. Several proteins were identified that have previously been characterized as having structural roles in cells. These proteins include actin (Nakayasu and Ueda, 1984), matrin 3 (Belgrader et al., 1991; Nakayasu and Berezney, 1991), lamin A/C (Jagatheesan et al., 1999), and pinin (Brandner et al., 1997; Ouyang et al., 1997; Ouyang and Sugrue, 1996). In

addition, our analysis identified several proteins that were recently identified by others has having roles in pre-mRNA splicing or to be localized in nuclear speckles (Liet al., 1999). These proteins include acinus (Manley and Reinberg, Blencow), the human homolog (KIAA0536) to yeast PRP4 (Kojima et al., 2001) as well as CrkRS kinase (Ko et al, 2001), eIF4Aiii (Holzmann et al., 2000). Most interestingly, we found 16 new proteins for which no available biological information is available except for sequence information. Each of these proteins was analyzed for known motifs.

In the last few years, much data has accumulated showing that various non-translatable non-coding RNA transcripts are present in different cell types. They are lacking a protein coding capacity and exert their functions mainly or exclusively at the RNA level. Such RNAs are known to play versatile roles in various aspects of nuclear function like, chromosome silencing, protein trafficking, act as a structural framework to nuclear domains (Eddy, 1999; Prasanth et al, 2000) and are even connected with well known developmental, neurobiological disorders and cancer (Askew and Xu, 1999; Volker et al, 2001). Work from several groups has pointed to the presence of such a population of stable poly A+ RNA in IGCs where they are hypothesized to serve as a structural scaffold of the IGCs (Cheniclete and Bendayan 1990; Thiry, 1993; Huang et al, 1994). In addition to examining the protein components of IGC from breast cancer cells, we also envisage understanding the intriguing link between this class of non-coding RNA/s, their role in maintaining the structural integrity of IGCs, and their characterization in carcinogenesis. As an initial part of this study we have purified RNA from IGCs and cDNA was synthesized using oligo dT primers, and a library was constructed. Work is underway to sequence and characterize the genes coding for the stable poly $A^{(+)}$ RNAs in the IGCs.

PURIFICATION OF IGCs FROM HUMAN CELL LINES: Before purifying IGCs from breast cancer cell lines, IGC purification was further standardized using a HeLa cell line because of the ease of procuring and grow them in suspension culture. Immunoblot analysis of various fractions of the HeLa IGC preparation clearly showed that the proteins that were enriched in mouse liver IGCs were also found to be enriched in HeLa IGC fractions (splicing factors and RNA processing factors) {See Fig1; Antibody against a splicing factor SF2/ASF clearly showed enrichment in IGC fraction in both mouse (fig.1A) as well as HeLa (Fig.1B) IGC preparations. Similar observations were made using 3C5 antibody that recoganize the SR family of splicing

factors (Fig.2A & B)}. For HeLa IGC purification the minimum number of cells necessary to begin IGC purification was found to be 6 X 10^9. The two cell lines (MCF-7 and HBL-100) that had been proposed previously (by previous PI) from which to purify IGC are generally grown in culture as attached cells. Thus, it would be an enormous task to grow these cell-lines at a density of 6 X 10^9. As an alternative we are presently trying to grow another breast cancer epithelial cell line HTB-123 as a suspension culture. This will allow us to achieve the cell density necessary for sufficient IGC purification. Work is currently underway to purify IGCs from this cell line. To our knowledge, there is no known non-cancerous breast cell line, which can be grown as suspension culture at such a high density. Thus it is nearly impossible to get a purified fraction of IGCs from a non-cancerous breast cell line. IGCs are believed to play important roles in coordinating transcription and splicing that are highly conserved throughout the eukaryotic phyla. As an alternative approach, to compare IGC protein composition between normal and cancerous cell lines we will have to limit ourselves to comparing the IGC fraction from normal mouse liver and the breast cancer cell lines. Once any factor/s that shows variation between IGCs of the breast cancer cell line to that of mouse liver is identified, it will be further confirmed in other non-cancerous breast cell lines by various *in situ* analyses. Using this approach we will be able to accomplish our goal of identifying the differences in the protein composition of IGCs between breast cancer cells and normal cells.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of identification of protein composition of interchromatin granule clusters from mouse liver nuclei.
- Based upon the above study, development of a standarized approach to purify IGCs from suspension tissue culture cells.
- Immunoblot analysis to confirm IGC fractionation from suspension cells.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have successfully identified the entire protein composition of the isolated IGC fraction from the mouse liver nuclei by proteome analysis using liquid chromatography

and tandem mass spectrometry (LC-MS/MS) and combining peptide sequence analysis and database searching. Interestingly, several proteins were identified with well characterized roles in pre-mRNA splicing and yet others known to have structural roles in cells. In addition, we have established the approach to purify IGCs from suspension tissue culture cells, HeLa. The quality of the IGC fraction from the suspension culture has been confirmed using immunoblot analysis. Further, it was observed that the proteins that were enriched in mouse liver IGCs were also found to be enriched in HeLa IGC fractions (splicing factors and RNA processing factors). Work is underway to purify and characterize the IGCs from the breast cancer cell line, HTB 123.

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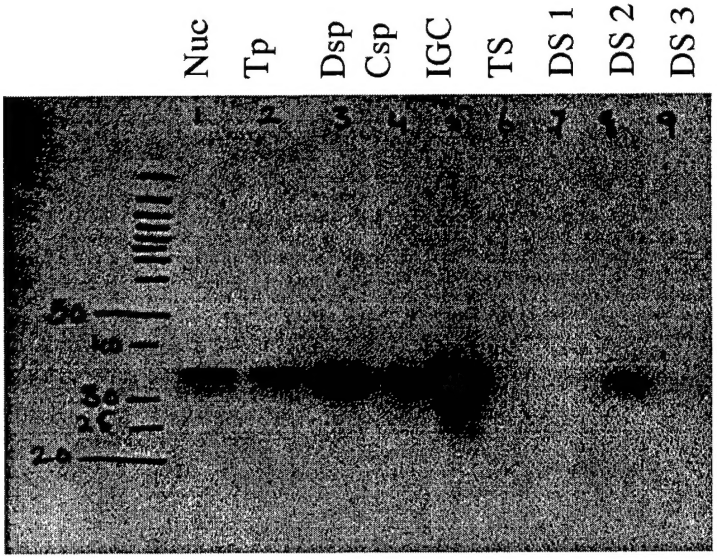
APPENDICES:

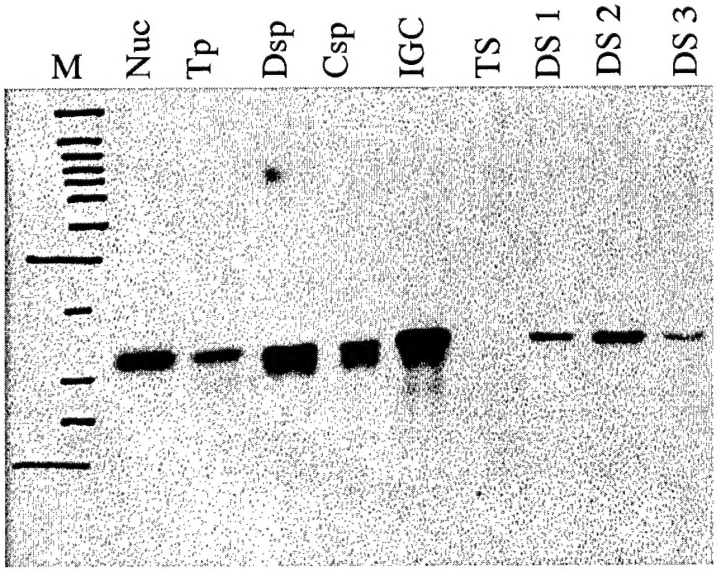
Figure 1 and 2 illustrating the enrichment of splicing factors in IGC fraction in mouse liver and HeLa cells

Fig.1A



Mouse liver

B

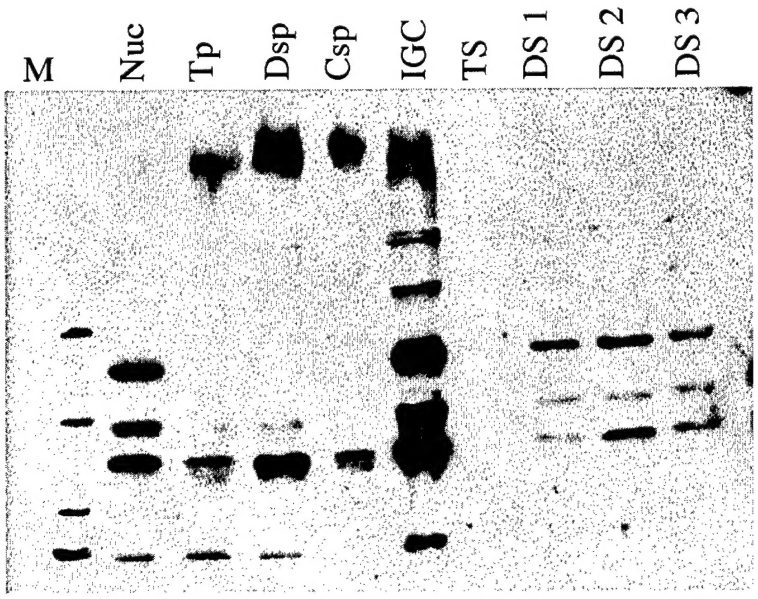


HeLa

Fig.1 Immunoblot analysis using an antibody that recognizes the splicing factor SF2/ASF in various fractions of IGC preparation from Mouse liver (Fig. 1A) and HeLa cells (Fig. 1B) clearly showed the enrichment in IGC fraction (lane 5 in both the blots).

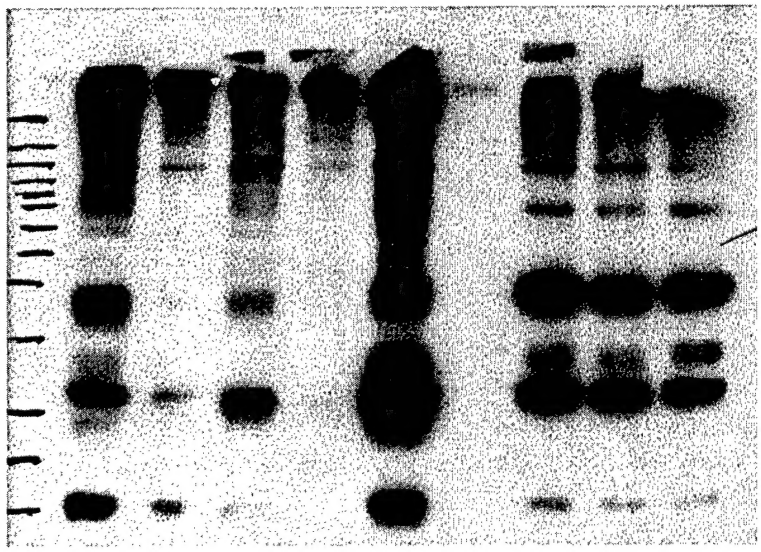
Nuc: Nuclei
Tp: Triton pellet
Dsp: DNase I fraction
Csp : Caesium pellet
IGC: IGC enriched fraction
TS: Triton X-100 supernatant
DS1-DS3: Salt washes

Fig.2A



Mouse liver

Fig.2B



HeLa

Fig.2 Immunoblot analysis using an antibody (3C5) that recognizes the phosphorylated form of SR splicing factors in various fractions of IGC preparation from Mouse liver (Fig.2A) and HeLa cells (Fig. 2B) clearly showed the enrichment in IGC fraction (lane IGC in both blots).

Nuc: Nuclei
Tp: Triton pellet
Dsp: DNase I fraction
Csp : Caesium pellet
IGC: IGC enriched fraction
TS: Triton X-100 supernatent
DS1-DS3: Salt washes